Heterologous expression and secretion of Clostridium perfringens [beta]-toxoid by Bacillus subtilis

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Chapter 5

Changing a single amino acid in *Clostridium perfringens* β-toxin affects efficiency of heterologous secretion by *Bacillus subtilis*

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ABSTRACT

Efficient heterologous protein production and secretion by *Bacillus subtilis* is an attractive prospect, although often disappointingly low yields are reached. The expression of detoxified *Clostridium perfringens* β-toxin (β-toxoid) is exemplary for this. Although β-toxin can efficiently be expressed and secreted by *Bacillus subtilis*, the genetically detoxified, and industrially interesting, β-toxoid variant is difficult to obtain in high amounts. To optimize the expression of this putative vaccine component, we studied the differences in global gene regulation response of *B. subtilis* to overproduction of either β-toxin or β-toxoid by transcriptomics. A clear difference was the upregulation of the CssRS regulon, known to be induced upon secretion stress, when β-toxoid was produced. YkoJ, a protein of unknown function, was also upregulated, and we show that its expression is dependent on *cssS*. We then focused on the heterologous protein itself and found that the major secretion bottleneck can be traced back to a single amino acid substitution between the β-toxin and the β-toxoid, which results in rapid degradation of β-toxoid following secretion across the cytoplasm membrane. In contrast to β-toxin, β-toxoid protein is more prone to degradation directly after secretion, most likely due to poor folding characteristics introduced with the point mutations. Our results show that, although the host can be adapted in many ways, the intrinsic properties of a heterologous protein can play a decisive role when optimizing heterologous protein production.

INTRODUCTION

Bacillus subtilis is widely used for protein production and secretion (Harwood, 1992). Nevertheless, secretion of heterologous proteins is often problematic, and many attempts have been made to overcome the poor production of secreted heterologous proteins, with varying success. A major problem encountered when using *Bacillus subtilis* as a production platform is protein degradation by extracellular proteases secreted by this organism. Using extracellular protease deficient strains this problem can be reduced to some extent, but such strains are more prone to lysis and have a reduced growth rate (Westers et al., 2004a).

Another bottleneck is the rapid degradation of the secreted protein by a quality-control mechanism in the cell wall environment (Stephenson et al., 1998; Bolhuis et al., 1999a; Jensen et al., 2000; Thwaite et al., 2002). Typically this degradation takes place before the protein can fold into its native and usually protease-resistant conformation. *B. subtilis* responds to the overexpression of secreted proteins with the so called secretion-stress response. This stress response is controlled by the CssRS two component system and regulates the expression of HtrA and HtrB, two serine proteases that also can act as chaperones (Darmon et al., 2002). Secretion stress is thought to be triggered by unfolded proteins at the trans side of the membrane due to problems that occur in late stages of protein secretion (Leloup et al., 1997), presumably as a consequence of slow folding at the membrane-cell-wall interface (Braun et al., 1999; Hyyrylainen et al., 2001).

To promote correct and rapid folding of the secreted heterologous proteins several measures can be taken. Expression of chaperones and proteases can be altered (Vitikainen et al., 2005), the charge of the cell wall or the secreted protein can be adapted (Stephenson et al., 2000), and the availability of divalent metal ions can accelerate folding of the proteins (Stephenson et al., 1998). However such measures usually improve secretion by only a factor 1.5 to 3.

In this work we report on important factors influencing the secretion of β-toxoid, a genetically inactive form of *Clostridium perfringens* β-toxin. This protein is of industrial interest since it is a major component in vaccine preparations protecting against *C. perfringens* type B and C infection. The wild type *C. perfringens* β-toxin is a potent toxin that requires chemical deactivation before it can be used as a safe vaccine component. Point mutations have been introduced that render this toxin no longer toxic but still immunogenic. However, this altered β-toxoid is very poorly secreted by *B. subtilis* (Nijland et al., 2007b).

In an attempt to identify the bottleneck in secretion of β-toxoid, we compared global gene expression during overproduction of β-toxin and β-toxoid. We tested if altering expression of the strongest upregulated gene could improve secretion yields. Unfortunately this did not yield the desired results.

Strikingly, the wild type β-toxin protein can be efficiently secreted by *B. subtilis*. We therefore focused on the protein itself and analyzed the specific effects of the amino acid substitutions that differ between β-toxin and β-toxoid. Surprisingly, this revealed that only a single amino acid residue dictates the difference between high and very poor secretion yields.

MATERIALS AND METHODS

Bacterial strains, medium and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown at 30°C in M17 broth with 0.5% glucose (GM17) (Terzaghi and Sandine, 1975). *B. subtilis* strains were grown at 37°C under vigorous agitation in TY (1% tryptone, 0.5% yeast extract, 1.0% NaCl) or minimal medium (Spizizen, 1958). For the selection of transformants, appropriate antibiotics were added to the growth media at the following concentrations: chloramphenicol 5 µg/ml, spectinomycin 100 µg/ml, hygromycin 125 µg/ml and erythromycin 5 µg/ml.

Strain constructions and transformation

The cloning and transformation procedures were performed according to established techniques (Bron and Venema, 1972; Sambrook et al., 1989b) and supplier's manuals. Restriction enzymes, DNA polymerases, deoxynucleotides and T4 DNA ligase were obtained from Roche Diagnostics (Mannheim, Germany) and Fermentas life sciences (Vilnius, Lithuania), and used as specified by the supplier. Table 2 lists the nucleotide sequences of primers used for PCR.

Table 1. strains and plasmids used in this study

Strains	Genotype	Reference
B. subtilis 168	*trpC2*	(Anagnostopoulos and Spizizen, 1961)
B. subtilis NZ8900	*trpC2*, amyE:: *spaRK*	(Bongers et al., 2005)
B. subtilis WB800	*nprE aprE epr bpr mpr*::*ble nprB*::*bsr* Δ*vpr wprA*::*hyg*	(Wu et al., 2002)
B. subtilis NZ8900-ΔykoJ	*trpC2*, *amyE:: SpaRK, ykoJ::SpcR*	This study
B. subtilis HT100A	NZ8900 containing P*htra*-GFP fusion integrated in the chromosome	This study
B. subtilis P*ykoJ-GFP*	NZ8900 containing P*ykoJ*-GFP fusion integrated in the chromosome	This study
L. lactis MG1363	Plasmid-free strain	(Gasson, 1983)
L. lactis NZ9000	*penN::NisRK*	(Kuipers et al., 1998)

Plasmids	Characteristics and description	Reference
pSG1151	*bla cat gfp*	(Lewis and Marston, 1999)
p*PykoJ*-GFP	pSG1151::P*ykoJ-gfp*	This study
p*PhtrA*-GFP	pSG1151::P*htrA-gfp*	This study
pDG1726	Vector containing spectinomycin resistance cassette	(Guerout-Fleury et al., 1996)
pXB10	pUB110 containing β-toxin coding region	(Steinthorsdottir et al., 1998)
pNZ8048	Nisin vector, Em^r	(de Ruyter et al., 1996)
pNZbtoxoid (pNZbtox)	pNZ8048::β-toxoid	(Nijland et al., 2007c)
pNZbtoxin	pNZ8048::β-toxin	This study
pNZ8901	Cloning vector containing subtilin inducible promoter, Cm^r	(Bongers et al., 2005)
pNZ8903	Cloning vector containing WT subtilin inducible promoter, Em^r	(Bongers et al., 2005)
pNRS-ykoJ	pNZ8901 containing ykoJ cds	This study
pNRS-βtoxoid (AAA)	pNZ8903 containing β-toxoid cds	This study
pNRS-βtoxin (DDK)	pNZ8903 containing β-toxin cds	This study
pNRS-βtox-DAA	pNZ8903 containing β-tox-DAA gene	This study
pNRS-βtox-ADA	pNZ8903 containing β-tox-ADA gene	This study
pNRS-βtox-AAK	pNZ8903 containing β-tox-AAK gene	This study
pNRS-βtox-DDA	pNZ8903 containing β-tox-DDA gene	This study
pNRS-βtox-DAK	pNZ8903 containing β-tox-DAK gene	This study
pNRS-βtox-ADK	pNZ8903 containing β-tox-ADK gene	This study
pRNΔ*ykoJ*_Sp	pUC18 containing up- and downstream regions of *ykoJ* flanking a spectinomycin resistance cassette	This study

Inducible β-toxin and YkoJ plasmids

To construct the *B. subtilis* subtilin-inducible plasmids pNRS-βtoxoid and pNRS-βtoxin the β-toxoid gene was amplified by PCR from the pBtox-1 plasmid and the β-toxin gene was amplified from plasmid pXB10, respectively, using primers Btox-fw_BstEII and Btoxoid-RV2-XhoI. These PCR products were digested BstEII, AvaI and ligated into the likewise digested replicative vector pNZ8903 containing the unmodified subtilin-inducible *spaS* promoter (Bongers et al., 2005). To construct pNRS-ykoJ the *ykoJ* gene was amplified by PCR from *B. subtilis* 168 chromosomal DNA using primers ykoJ-fw1 and ykoJ-rv1, digested BstEII, AvaI and ligated into the likewise digested replicative vector pNZ8901 containing the subtilin-inducible *spaS* promoter (Bongers et al., 2005). To construct the *L. lactis* plasmids pNZ-βtoxoid and pNZ-βtoxin the β-toxoid gene was amplified by PCR from the pBtox-1 plasmid and the β-toxin gene was amplified by PCR from plasmid pXB10 using primers Btoxoid-RN2-fw and Btoxoid-RN2-rv. This product

was digested with NcoI, AvaI and ligated in the likewise digested replicative vector pNZ8048 containing the nisin-inducible promoter (de Ruyter et al., 1996). Ligation mixtures were transferred to electro-competent *L. lactis* MG1363 or *L. lactis* NZ9000 using a Gene pulser (Biorad laboratories, Hercules, California), as described earlier (Leenhouts et al., 1989). Colonies were selected on solid media for the erythromycin resistance. Isolated plasmids were checked for correct ligation by AvaI-AvaII digestion and DNA-sequencing.

B. subtilis NZ8900 was transformed with the constructed replicative plasmids isolated from *L. lactis*, and selected on solid medium for appropriate resistance.

Table 2. Primers used in this study

Primer name	Description	Sequence (5' to 3')[a]
Btoxoid-RN2-fw	Forward primer for β-toxoid cloning in pNZ8048	CGTTGCCATGGAGAAAAAATTTATTTCATTAGTTATAG
Btoxoid-RN2-rv	Reverse primer for β-toxoid cloning in pNZ8048	CGCTCTAGATTAAATAGCTGTTACTTTGTGAG
Btox-fw_BstEII	Forward primer for Btoxoid_SS introduces BstEII and NdeI sites	TCGGTGACCCATATGAAGAAAAAATTTATTTCATTAG
Btoxoid-RN2-XhoI	Reverse primer for β-toxoid, introduces XhoI site	CGCCTCGAGTTAAATAGCTGTTACTTTGTGAG
RNlacZ-fw	Universal primer on pUC18 derived MCS	GTGAGCGGATAACAATTTCACACAGG
RNlacZ-rv	Universal primer on puc18 derived MCS	GGTTTTCCCAGTCACGACGTTGTAA
PhtrA_fw-KpnI	Forward primer for promotor region *htrA*, introduces KpnI site	CGTGAGGTACCGGCTTCTGTTTCTGCC
PhtrA_rv	Reverse primer for promotor region *htrA*, introduces HindIII site	CATCACGAAGCTTATCCATCATGTTCACTCCG
PykoJ-fw1	Forward primer for amplification of *ykoJ* promoter, introduces KpnI site	CTGGTACCGCAGTGAATCCATCTGCCATGAC
PykoJ-rv1	Reverse-primer for amplification of *ykoJ* promoter, introduces HindIII site	ACTAAGCTTGAGCATTTGTGAGCCCTCCTTTGT
ykoJ-fw1	Forward primer to amplify *ykoJ* for overexpression, introduces BstEII-site	TGTGGTGACCCAAATGCTCAAGAAAAAATGGATGGTCGGTCTTTTAG
ykoJ-rv1	Reverse primer to amplify *ykoJ* for overexpression, introduces XhoI-site	ACACTCGAGTTAGTCATCTATCTCCTGTTTGATAATG
up_ykoJ-fw	Forward primer to amplify upstream region of *ykoJ* internal pstI-site is amplified	CTTTTGCTGCAGCAGCCATTTTAG
up_ykoJ-rv	Reverse primer to amplify upstream region of *ykoJ,* introduces HindIII-site	TCTTAAGCTTTTGTGAGCCCTCCTTTGTTT
down_ykoJ-fw	Forward primer to amplify downstream region of *ykoJ,* introduces HindIII site	TCTAAGCTTCAGGAGATAGATGACTAATCAA
down_ykoJ-rv	Reverse primer to amplify downstream region of *ykoJ,* introduces XbaI site	CGATCTAGAAGCATCCAGCTGCATTA
btox-change_FW	universal forward primer for mutagenisis of β-toxin , introduces Eco31I site	CGGTCTCATATTCATCTGAAATGACAACTTTAATAAACTTAAC

RV-btox_DAA	Reverse primer change DDK --> DAA	CGGTCTCAAATAAGCAGCATCGATA AATCTAGCATCTATAGATGCAGTAA
RV-btox_ADA	Reverse primer, changes DDK --> ADA	CGGTCTCAAATAAGCATCAGCGATA AATCTAGCATCTATAGATGCAGTAA
RV-btox_AAK	Reverse primer, changes DDK --> AAK	CGGTCTCAAATATTTAGCAGCGATA AATCTAGCATCTATAGATGCAGTAA
RV-btox_DDA	Reverse primer, changes DDK --> DDA	CGGTCTCAAATAAGCATCATCGATA AATCTAGCATCTATAGATGCAGTAA
RV-btox_DAK	Reverse primer, changes DDK --> DAK	CGGTCTCAAATATTTAGCATCGATA AATCTAGCATCTATAGATGCAGTAA
RV-btox_ADK	Reverse primer, changes DDK --> ADK	CGGTCTCAAATATTTATCAGCGATA AATCTAGCATCTATAGATGCAGTAA

ykoJ gene deletion construct

Upstream and downstream regions of the *ykoJ* gene were amplified by PCR using primers up_ykoJ-fw1, up_ykoJ-rv1 and down_ykoJ-fw1, down_ykoJ-rv1 respectively, and resulting PCR products were digested PstI + HindIII, HindIII + XbaI respectively. These products were ligated in a four point ligation to both sides of a HindIII digested spectinomycin resistance cassette, obtained by PCR using pDG1726 as a template and primers RNlacZ-fw and RNlacZ-rv, and a PstI + XbaI digested pUC18. The resulting plasmid pRNΔykoJ_Sp was amplified in *E. coli* and transformed to *B. subtilis* NZ8900 to create NZ8900-ΔykoJ. Colonies were checked for integration of the spectinomycin resistance cassette via double crossover at the locus of *ykoJ* by PCR.

Intermediate β-toxin mutants

All β-toxin variants were constructed by round-PCR on the template plasmids pNRS-βtoxin (to create pNRS-βtox-A_{54}DK, -A_{54}DA, -A_{54}AK) and pNRS-βtoxoid (to create pNRS-βtox-D_{54}AK, -D_{54}DA, -D_{54}AA). The plasmids were amplified using a forward primer annealing next to the mutagenesis target site and containing an Eco31I recognition site, and a specific reverse primer containing the desired point mutation and an Eco31I site (see Table 2). After amplifying the whole plasmid the PCR product was digested with Eco31I and circularized by self-ligation. The resulting plasmid was electroporated to *L. lactis* MG1363. Mutants were checked for the appearance/disappearance of the ClaI site contained within the first codon of the DDK region, and subsequently checked by DNA sequencing (Baseclear, Leiden, NL). Correctly constructed plasmids were transformed to *B. subtilis* NZ8900 and selected for erythromycin resistance.

Protein expression, protein isolation, gel electrophoresis and Western blotting

B. subtilis cultures were diluted from an overnight culture to a starting OD_{600} of 0.1. β-toxin or β-toxoid expression was induced when the culture reached an OD_{600} of ~0.5 by the addition 0.75% of subtilin containing supernatant of strain ATCC6633 prepared as described earlier (Bongers et al., 2005). Two hours after induction cells were separated from the supernatant by centrifugation for 1 minute at 14,000 rpm. Supernatant proteins were concentrated 20-fold following TCA precipitation and prepared for SDS-page as described previously (Laemmli, 1970). Cell fractions were prepared for SDS-PAGE as described previously (Veening et al., 2004). Proteins were separated by SDS-PAGE, and either stained with Coomassie Brilliant Blue directly, or transferred to a polyvinylidene-difluoride (PVDF) membrane (Molecular Probes Inc., Eugene, Oregon). β-toxoid protein was visualized using a monoclonal anti-β-toxoid antibody (Intervet Int., Boxmeer, NL), and a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Amersham Biosciences, Buckinghamshire, UK). Protein size and concentrations were determined with a prestained protein marker (Fermentas, Vilnius, Lithuania), and the Quantity One software (Biorad, Hercules, California).

DNA-microarray experiments

DNA microarray procedures were performed as described by Lulko et al, 2006 (in press). In short, RNA was isolated from three independently grown cultures of *B. subtilis* NZ8900 containing either pNRS-βtoxin or pNRS-βtoxoid. β-toxin or β-toxoid expression was induced as described above and samples for RNA-isolation were taken 1.5h after induction with subtilin. Single-strand reverse transcription (amplification) and indirect labeling of total isolated RNA with either Cy3 or Cy5 dye were performed and labeled cDNA samples were hybridized O/N at 48°C on in-house printed micro-array slides containing 70-meric oligo's covering all *B. subtilis* open reading frames. After hybridization, slides were washed and scanned. Slide data were processed and normalized as described previously (den Hengst et al., 2005), yielding average ratios of gene expression levels of the strain expressing the β-toxoid compared to the strain expressing the WT β-toxin. Expression of a gene was considered to be significantly altered when its expression ratio was >1.75 or <0.57 and had a CyberT Bayesian P value of <0.001. All DNA microarray data obtained in this study are available online. (http://molgen.biol.rug.nl/publication/btox_data/)

P*htrA*-GFP and P*ykoJ*-GFP analysis

The *htrA* and *ykoJ* promoter regions were amplified by PCR using primers PhtrA-fw-kpnI, PhtrA-rv and PykoJ-fw, PykoJ-rv respectively. The PCR products were digested HindIII, KpnI and ligated in the likewise digested plasmid pDG1151. The plasmids were transferred to *E. coli* and correct clones were checked by PCR and DNA sequencing. The pP*htrA*-GFP plasmid was integrated via single crossover in the chromosomal DNA of *B. subtilis* strain NZ8900 at the locus of the *htrA* promoter creating strain *B. subtilis* HT100A and the pP*ykoJ*-GFP plasmid was likewise integrated at the locus of the *ykoJ* promoter creating strain *B. subtilis YkoJ*-GFP. GFP production was measured using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijndrecht, NL). Average fluorescence of 20.000 gated cells was determined using WinMDI 2.8 (http://facs.scripps.edu/software.html).

Assay on β-toxin and β-toxoid stability

An O/N culture of *L. lactis* NZ9000 containing either pNZβtox or pNZβtoxin was diluted to OD_{600} 0.1 and grown for 2.5h until OD600 of 0.5. Nisaplin (stock 50mg/ml) in a final dilution of $1x10^{-7}$ was added to induce the nisin-inducible promoter, and 2 hours after induction total supernatant was harvested by centrifugation and subsequent filtration over a 0.2µm syringe-filter (Schleicher and Schuell microscience, Dassel, Germany).

To collect spent supernatant of *B. subtilis* strains 168 and WB800, the strains were grown in TY and supernatant samples were taken 2 hours into stationary growth phase. Supernatant was separated by centrifugation and subsequently passed trough a 0.2µm filter. The β-toxin and β-toxoid samples harvested from *L. lactis* were mixed 1:1 with the *B. subtilis* spent supernatant and incubated for 10 minutes and 1h at 37°C. As a control fresh TY was used. After incubation total protein was concentrated 10x upon TCA precipitation as described before and analyzed using SDS-PAGE. Concentration of β-toxin and β-toxoid was determined by Coomassie Brilliant Blue staining followed by densiometric scanning (Biorad GS-800 scanner) and analysis with Quantity one software (Biorad, Hercules, California).

RESULTS

Recently, we have described the difficulties in secretion of *Clostridium perfringens* β-toxoid by *Bacillus subtilis* (Nijland et al., 2007c). Yet, it has been reported that wild type β-toxin protein can be efficiently expressed in *B. subtilis* (Steinthorsdottir et al., 1998). To directly compare the secretion efficiency of both proteins, we cloned them in the subtilin-inducible SURE vector pNZ8903. After induction for 1.5h cells were separated from the growth medium and β-toxin and β-toxoid production and secretion

were assayed by SDS-PAGE and Western blotting using monoclonal anti-β-toxin antibody (Fig. 1). Clearly, the β-toxin protein was secreted in high amounts, up to 50% of total extracellular protein, whereas the β-toxoid variant never yielded more than 4%. The intracellular levels of β-toxin and β-toxoid were similar and both induced cultures showed no significantly decreased growth rate compared to uninduced cultures. Furthermore, no significant growth difference between the β-toxin and β-toxoid production strains was observed (data not shown).

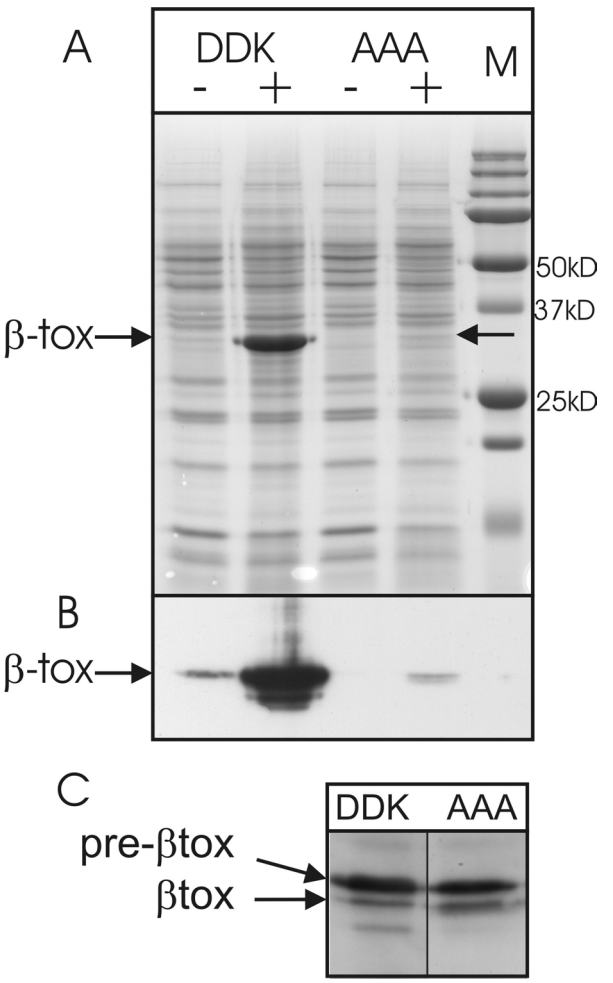

Figure 1. Production and secretion of β-toxin and β-toxoid

A: Coomassie brilliant blue stained 12% SDS-PAGE gel containing 10x concentrated supernatant of *B. subtilis* strain NZ8900 1,5h after induction of the inducible β-toxin/β-toxoid plasmids.

B: Detection of secreted β-toxin and β-toxoid in the samples show in panel A, examined by Western blotting using monoclonal antibodies against β-toxin.

C: Detection of intracellular β-toxin and β-toxoid examined by Western blotting using monoclonal antibodies against β-toxin. Pre-βtox and βtox are indicated by the arrows. DDK = WT β-toxin (D_{54}DK); AAA = β-toxoid (A_{54}AA). M = protein marker. + = induction with subtilin; - = no subtilin added.

Global gene expression differences between β-toxin and β-toxoid overproduction

From Fig. 1 it is apparent that there is a large difference in secretion yield between β-toxin and detoxified β-toxoid. To identify possible bottlenecks in the secretion of β-toxoid, we compared the two production strains using DNA microarrays. Either β-toxin or β-toxoid expression was induced and samples for RNA-isolation were taken 1.5h after induction. Following statistical analysis of the obtained data, several genes showed a clear difference in expression levels between the two strains. An overview of the results of the DNA microarray is presented in Table 3.

Table 3. DNA microarray results

Gene	Ratio	Bayesian p-value	(putative) function
Upregulated genes, higher expression if β-toxoid expression is compared to β-toxin expression			
ykoJ	6.87	2.25E-14	Unknown
yvtA	2.66	1.47E-10	= *htrB*, serine protease, secretion stress
htrA	2.39	6.06E-10	Serine protease, secretion stress
spoIIR	2.33	0.000902	Sporulation
ykzD	2.28	5.95E-05	Unknown, directly downstream of *ykoJ*
yvqH	2.09	3.12E-06	= *liaI*, part of liaRS regulon
cggR	1.97	0.000437	Transcriptional repressor of gapA
yvqI	1.95	2.15E-05	= *liaH*, part of *liaRS* regulon
ldh	1.92	0.000111	L-lactate dehydrogenase
mtlD	1.92	6.48E-06	PTS, mannitol-specific enzyme
mtlA	1.88	2.57E-06	PTS, mannitol-specific enzyme
cssR	1.76	0.000847	Two component regulator, secretion stress
Downregulated genes, lower expression if β-toxoid expression is compared to β-toxin expression			
purN	-2.61	6.84E-11	Purine biosynthesis
purM	-2.55	1.06E-05	Purine biosynthesis
purH	-2.47	1.49E-08	Purine biosynthesis
rocR	-2.12	1.89E-08	Regulator, binding box upstream ykoJ
yomM	-1.94	0.00081	Unknown
yddE	-1.9	3.65E-05	Unknown, operon on transposon region
yddB	-1.86	3.72E-05	Unknown, operon on transposon region
yecA	-1.85	0.000455	Transport/binding proteins and lipoproteins
ydcP	-1.8	0.000163	Unknown, operon on transposon region
yosP	-1.79	0.000742	Unknown, operon on transposon region
yddF	-1.77	0.0003	Unknown, operon on transposon region

The strongest upregulated gene in the β-toxoid expressing strain was *ykoJ*, a gene of unknown function. Further *htrA* and *htrB* were upregulated, as well as *cssR,* part of the two component system known to regulate *htrA* and *htrB* expression (Darmon et al.,

2002). An upregulation of *htrA* upon expression of β-toxoid, as monitored by a P*htrA*-lacZ fusion, was previously described (Nijland et al., 2007c). Clearly, this upregulation is lower for the wild type β-toxin, since a difference in *htrA* expression is found in the array comparison. An overview of the other differentially expressed genes and their (putative) function is given in Table 3.

To validate the DNA microarrays we looked specifically at the expression of *ykoJ* and *htrA* by using promoter-GFP reporter fusions. We measured the average GFP expression per cell using flow cyctometry. As shown in Fig. 2A, *htrA* expression in a strain that expressed β-toxoid was 3 times higher compared to a strain that expressed β-toxin. It should be noted that without induction the P*htrA*-gfp levels are much lower, indicating that expression and secretion of β-toxin also causes secretion stress. As shown in Fig. 2B, the results obtained with a P*ykoJ*-gfp fusion confirmed the transcriptome result as well. Like *htrA*, P*ykoJ* was moderately upregulated when β-toxin was induced and strongly upregulated when β-toxoid was induced.

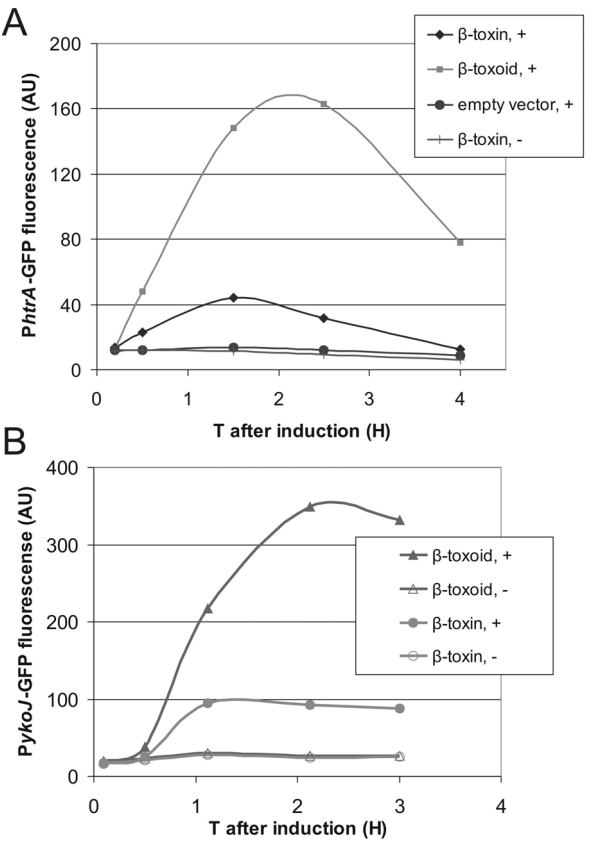

Figure 2A. P*htrA*-GFP upon overexpression of β-toxin and β-toxoid

Internal GFP fluorescence in time of *B. subtilis* HT100A containing either β-toxin of β-toxoid on an inducible plasmid. After induction at T_0 of β-toxin/β-toxoid the response of the *htrA* promoter was measured by quantifying average GFP fluorescence per cell using a flow-cytometer.

Figure 2B. P*ykoJ*-GFP upon overexpression of β-toxin and β-toxoid

Internal GFP fluorescence in time of *B. subtilis* P*ykoJ*-GFP in its chromosome and either β-toxin of β-toxoid on an inducible plasmid. After induction at T_0 of β-toxin/β-toxoid the response of the *ykoJ promoter* was measured by quantifying average GFP fluorescence per cell using a flow-cytometer. Also not induced cultures were measured. + induced; Figure 2A - not induced.

Since the expression of *ykoJ* resembled the expression pattern of *htrA,* we tested whether the expression of *ykoJ* was controlled by the CssRS two component system. For this a *cssS* disruption (Hyyrylainen et al., 2001) was introduced into the P*ykoJ*-gfp reporter strain. As shown in Fig. 3, no response of the *ykoJ*-promoter occurred upon induction of β-toxin or β-toxoid when *cssS* was mutated. Together, the microarray analysis data show that the induction of the secretion stress regulon governed by CssRS is most apparent upon induction of β-toxoid.

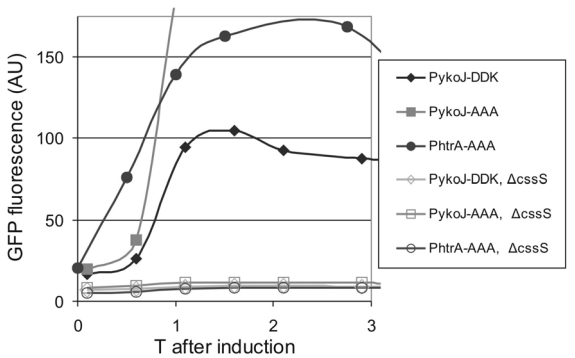

Figure 3. Effect of *cssS* disruption on response of P*ykoJ*-GFP and P*htrA*-GFP

Internal fluorescence was measured of *B. subtilis* HT100A or P*ykoJ*-GFP. Response was determined with or without a *cssS* disruption in the strain. In all strains β-toxin (D_{54}DK) or β-toxoid (A_{54}AA) was induced by the addition of subtilin at T_0.

YkoJ deletion does not improve β-toxoid secretion

The transcriptome data suggests that the CssRS regulon could be a target when improving secretion of β-toxoid. In a previous study we have already shown that mutation of the CssRS two component system does not positively effect production of β-toxoid (Nijland et al., 2007c). Since a mutation of CssRS will effectively prevent induction of HtrA and HtrB (Darmon et al., 2002), deletion of these two induced proteases separately is unlikely to improve secretion of β-toxoid. In studies performed by Vitikainen et al. (2005) it was also observed that downregulation or mutation of HtrA and/or HtrB proteases does not improve secretion but instead induces severe stress to the cells resulting in poor growth and generally lower secretion yields. However, since *ykoJ* was strongly overexpressed in our study, we tested whether this protein itself influences the efficiency of secretion of β-toxoid. YkoJ contains two PepSY domains that suggest a peptidase inhibiting action (Yeats et al., 2004), but the specific function of YkoJ is still unknown. A deletion of *ykoJ* showed no noteworthy improvement of β-toxoid secretion. Also the secretion of β-toxin in this strain did not differ from the wild type strain (data not shown). We constructed a YkoJ overproduction strain using the SURE expression system. Unfortunately, upon mild induction, the cultures stopped growing and started lysing, indicating that the overexpression of *ykoJ* is lethal to *B. subtilis* (data not shown). These results indicate

that YkoJ alone is not directly involved in the large difference in secretion between β-toxin and β-toxoid.

Amino acid differences between β-toxin and β-toxoid

Since altering the expression of host genes did not improve the yield of secreted β-toxoid, we focussed on the nature of the protein itself. The differences between β-toxin and β-toxoid are three consecutive mutations at the N-terminal side of the mature protein (D54A, D55A, K56A). Based on a homology model of the mature β-toxin protein available at the MODBASE protein model database (Pieper et al., 2004), we have looked at the position of these residues in the folded protein.

According to the model, β-toxin consists largely of β-sheets. However, the residues 54, 55, and 56 (as counted from the first residue of the mature protein) are situated in a loop at the surface of the protein, and consist of two negatively charged aspartic acids and a positively charged lysine. In β-toxoid these residues are replaced with alanines. Likely this change in charge distribution affects the folding characteristics of the protein.

Since the amino acid substitutions in the β-toxin mutants might influence folding and stability of the protein, we tested susceptibility to proteases. β-toxoid and β-toxin produced by *L. lactis* were incubated with spent supernatant of a stationary *B. subtilis* culture. This culture supernatant contains many proteases secreted by *B. subtilis.* As shown in Fig. 4, a clear difference between the stability of both proteins is visible. Whereas more than 50% of the β-toxin is still present after one hour incubation, almost all β-toxoid (>90%) has been degraded. As a control we tested supernatant from *B. subtilis* WB800. In this strain the genes of 8 proteases have been deleted (Wu et al., 2002). Incubation with supernatant from a WB800 culture gave significantly less degradation and about 60% β-toxoid was still detectable after one hour incubation (Fig. 4). The results show that β-toxoid is more prone to degradation than β-toxin, indicating that the amino acid substitutions do make the protein conformation less stable.

Intermediate mutants between β-toxin and β-toxoid

To examine the importance of the individual amino acid mutations for protein secretion, we constructed all possible six intermediate mutants. Expression and secretion of the different β-toxin variants was tested by harvesting cells and supernatant fractions 1.5h after induction and analyzed by SDS-page and Western blotting (Fig. 5A). From these experiments two classes emerged, those with β-toxin production levels (D_{54}DK (=β-toxin), D_{54}DA, A_{54}DA, A_{54}DK), and those with hardly any protein secreted (A_{54}AA (=β-toxoid), A_{54}AK, D_{54}AK, D_{54}AA). This screening indicated that mutations at residues 54 and 56 do not have a significant effect on secretion efficiency, yet residue D_{55} is pivotal when it comes to efficient secretion of toxin.

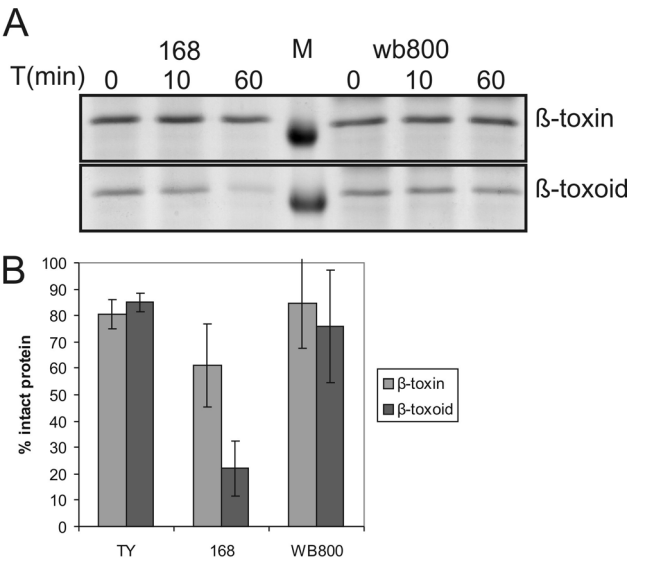

Figure 4. Degradation of β-toxin and β-toxoid by spent *B. subtilis* supernatant

Lactococcus lactis produced β-toxin and β-toxoid were incubated with spent supernatant of stationary phase culture of *B. subtilis* strains 168 and WB800. As a control TY was used.

A: Typical CBB stained PAA-gel showing results of degradation assay. Incubation time in minutes is indicated. Left lanes show β-toxoid and β-toxin exposed to 168 culture supernatant, right lanes show β-toxoid and β-toxin exposed to WB800 culture supernatant. M: protein marker, 35kD band.

B: Amount of β-toxin / β-toxoid measured after 10 minutes was set to 100%. Remaining β-toxoid and β-toxin after 1h was determined and plotted. Experiment performed in duplo, error bars depict standard error.

The mean lethal dose (MLD) of the β-toxin ($D_{54}DK$) and the β-toxoids $D_{54}DA$, $A_{54}DA$ and $A_{54}DK$ were assessed by intravenous injection, in sterile PBS, into mice of approximately 25g. All three β-toxoids had a similar level of toxicity which is approximately one fifth of that of the toxin. The original β-toxoid ($A_{54}AA$) has a toxicity that is approximately 30 times lower than the wild type toxin (Segers et al., 1999). The poorly secreted β-toxoids ($A_{54}AK$, $D_{54}AK$, $D_{54}AA$) were not tested since production levels were to low.

We also measured the effect on *htrA* expression with these 6 intermediate mutants. In accordance with the previous results all mutants with an aspartic acid at position 55 showed relative low, β-toxin like, *htrA* expression levels, whereas the mutants with an alanine at this position showed a strong upregulation of *htrA*, comparable to that for β-toxoid production (Fig. 5B). Clearly there is a strong relation between poor β-toxoid secretion and the induction of the secretion-stress regulon.

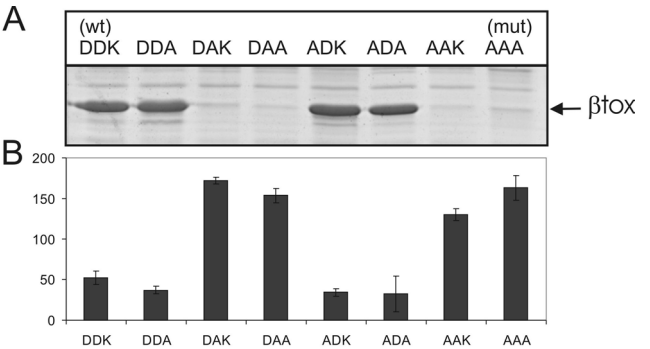

Figure 5. Secretion and secretion stress of β-toxin, β-toxoid and intermediate mutants

A: CBB stained 12% SDS-PAGE gel containing 10x concentrated supernatant of *B. subtilis* strain NZ8900 1.5h after induction of the inducible β-tox plasmids.

B: Average P*htrA*-GFP expression per cell in arbitrary units, measured 1.5h after induction. Experiment performed in duplo, error bars depict standard error. DDK = WT β-toxin (D_{54}DK); AAA = β-toxoid (A_{54}AA) all other intermediate mutants are likewise indicated.

DISCUSSION AND CONCLUSION

β-toxoid, the genetically altered variant of *Clostridium perfringens* β-toxin is not efficiently produced by *Bacillus subtilis*. To improve the secretion yield of the β-toxoid protein we have swapped signal sequences, used several expression systems and tested protease deficient hosts (Nijland et al., 2007c). None of the tested methods resulted in an appreciable increase in yield. However, wild type β-toxin could be secreted much better than the β-toxoid, in yields exceeding 50% of total secreted protein fraction. To identify the bottleneck that was causing this difference in secretion yield, we applied a genome wide expression analyses of the two production strains, hoping to find genes or processes responsible for this large production difference.

The DNA microarray analysis revealed that the differences can be largely attributed to the CssRS regulon, an indication of unfolded protein stress. The most upregulated gene in our array study, *ykoJ*, appeared to be part of the CssRS regulon as well. A deletion of the CssS sensor, effectively preventing induction of *htrA*, *htrB* (Darmon et al., 2002) and *ykoJ* did not improve secretion. We tried to overproduce YkoJ but this proved to be lethal. Next to the CssRS regulon two genes present in the *liaRS* regulon (Jordan et al., 2006) were expressed significantly higher in the β-toxoid mutant. This effect was also found in another secretion stress study (Antelmann et al., 2003). Recently, it has been shown that LiaRS is activated by cell envelope stress (Jordan et al., 2006). Only *liaI* and *liaH*, the genes that are generally much higher expressed than the other genes in the regulon (Jordan et al., 2006), were significantly

upregulated in our study. The other genes that are part of this regulon were not found, indicating that the LiaRS induction differences are minor in our transcriptome comparison. We therefore did not characterize the effect of *liaRS* on β-toxoid production. Several of the purine biosyntheses genes were found down-regulated, indicating a slight decrease in growth rate, which was missed in the growth-rate determination, but is picked up by the more sensitive microarray analysis.

Since altering the production host to increase secretion of β-toxoid was so far not successful, we looked more closely at β-toxoid itself, as the difference in yield between β-toxin and β-toxoid is striking. The stretch of 3 amino acid substitutions that morphs β-toxin into β-toxoid are not located in the secretion signal peptide where point mutations can have large effects on secretion efficiency (Zanen et al., 2005). Furthermore, intracellular retention of both the β-toxin and the β-toxoid is similar, indicating that no stalling problems occur when the protein gets secreted over the cytoplasm membrane via the Sec translocon.

Proteins secreted via the Sec-secretion pathway are generally thought to be secreted in an unfolded state and are only folded after secretion over the plasma membrane (Tjalsma et al., 2004). The current model of the β-toxin protein suggests that the introduced point mutations in β-toxoid might interfere with the correct folding or rate of folding of β-toxoid after secretion. Upon induction of β-toxoid a secretion stress response is observed, most likely induced by unfolded, secreted protein (Braun et al., 1999; Hyyrylainen et al., 2001). These results suggest that β-toxoid is reaching the outside of the membrane. The exact signal that is sensed by the CssS secretion stress sensor is as yet unknown, as it could also be the breakdown-products of the malfolded and degraded protein that trigger the system.

The changed residues in β-toxoid are most likely effecting optimal folding kinetics and therefore the β-toxoid protein is much more prone to degradation. Our experiments validated this assumption and showed that β-toxoid was much more prone to proteolysis than β-toxin, indicating that β-toxoid is in a folded conformation that is less stable that the WT β-toxin. The tested β-toxoid was produced and secreted by *L. lactis*, which could have influenced the folding of this protein. However, this is likely to be equally true for the β-toxin which also was produced by *L. lactis*, which justifies this comparison. Incubation with the supernatant of a *B. subtilis* WB800 strain, which lacks the genes for seven extracellular proteases and the cell wall protease WprA, resulted in considerably less breakdown of β-toxoid. However, expression of β-toxoid by *B. subtilis* WB800 resulted only in a minimal improvement of β-toxoid secretion (Nijland et al., 2007c). This demonstrates that in the case of β-toxoid most of the secreted protein is degraded before it is targeted by WprA or the other extracellular proteases deleted in WB800.

The constructed intermediate mutants of β-toxin demonstrate that only the aspartic acid at position 55 is necessary for the high secretion of the β-toxin. Although

also residues 54 and 56 are charged and locate at the outside of the protein they seem to be unimportant for secretion efficiency. They do play a role in toxicity of the β-toxin, since the alterations of these residues does lowers toxicity five fold. The reason for this we do not know, possibly future structural studies might clarify this.

The response of the *htrA* and *ykoJ* promoters to overproduction of β-toxoid is indicative of extracellular folding stress. As proposed by Westers et al. (Westers et al., 2004a), the expression of P*htrA* or P*htrB* can be utilized to monitor protein secretion. This study has added the *ykoJ* promoter to the possible indicators of secretion stress. A screening method using this promoter and site directed/random mutagenesis of the secreted substrate should provide a rapid method to improve heterologous protein secretion.

With this study we present a case where the bacterial host can be adapted in many ways without a significant yield improvement of secreted heterologous protein. The bottleneck turned out to be the secreted protein itself, where one point mutation made a crucial difference. In many cases the intrinsic properties of the heterologous protein can be a main cause of the limited production yields, and increased attention to optimizing the protein itself rather than only the expression host is required.

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